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Identification of an embryonic isoform of myelin basic protein that is expressed widely in the mouse embryo

(development/gene expression/reverse transcriptase–polymerase chain reaction)

PETER M. MATHISEN, STEPHANIE PEASE, JUSTINE GARVEY, LEROY HOOD*, AND CAROL READHEAD†

Division of Biology 147-75, California Institute of Technology, Pasadena, CA 91125

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ABSTRACT **We have identified a myelin basic protein (MBP) isoform in mouse embryos that includes an exon upstream of the usual transcription initiation site. This isoform, embryonic–neonatal MBP (E-MBP), is expressed at the protein level in the embryonic nervous system at a time when other MBP isoforms are not detected. In addition to the central and peripheral nervous systems of the embryo and neonate, the thymus, spleen, and testes also express E-MBP at the protein level. The expression of E-MBP in cell types distinct from the nervous system strongly suggests that this MBP isoform has a role apart from the formation of myelin.**

During the development of the mammalian nervous system, myelination of neuronal axons is accomplished by specific glial cells, oligodendrocytes and Schwann cells, which coordinately regulate the expression of a unique set of genes that are required for the synthesis of the myelin sheath (1). Each oligodendrocyte, derived from the neuroepithelium, myelinate a number of internodes in the central nervous system (CNS), while each Schwann cell developed from the neural crest, myelinates a single internode of an axon of the peripheral nervous system (PNS). Between oligodendrocytes and Schwann cells there is expression of an overlapping set of myelin-specific genes that are particularly interesting because they are activated in different cell types that have unique cell lineages. One member of this class of genes, myelin basic protein (MBP), is required for normal CNS myelinogenesis, since an autosomal recessive mouse mutation, shiverer (*shi/shi*), a deletion of about 60% of the MBP gene, leads to severe hypomyelination of the CNS (2). Normal compact myelin is seen in transgenic shiverer mice, which have integrated the wild-type MBP gene (26).

In the mouse, the primary MBP transcript is alternatively spliced by the removal of specific exons to produce mRNAs encoding at least five different protein isoforms (3–5). An additional minor MBP mRNA isoform has been isolated by cDNA cloning of PCR products; however, it is not known whether this mRNA is actually translated into a 19.7-kDa protein (6). It has also been reported that several additional putative exons are located 5′ to the previously defined MBP gene (7, 8, 23). MBP gene expression has been detected at about postnatal day 7, peaking at about 18 days and decreasing to steady-state levels at about 30 days. In the PNS MBP mRNA is also detected postnatally.

Thus, MBP gene expression occurs specifically in two distinct cell types with two separate lineages in both a temporally and spatially regulated manner to produce gene products that are essential for the normal development and function of the nervous system. We used a sensitive and semiquantitative reverse transcriptase (RT)–PCR assay (9–11) to measure the expression of the various isoforms of MBP mRNA during development of the CNS and PNS. We found that MBP gene expression begins much earlier than has been measured previously. We also identified an embryonic–neonatal-specific isoform (E-MBP) that is expressed not only in the developing CNS but also in a number of other embryonic tissues as both mRNA and protein. This isoform contains at least one novel exon from the promoter region of the MBP gene. The expression of this isoform in nonmyelinating cell types suggests an additional function for the E-MBP isoform apart from a role in myelinogenesis.

MATERIALS AND METHODS

Mice. Embryos and neonates were collected from naturally mated BALB/c mice. The day of plug was embryonic day 0 (E0).

cDNA Synthesis and PCR Amplification. Total RNA was isolated from brains or sciatic nerves of staged mice with guanidine isothiocynate and CsCl (12, 13). Brain cDNA was synthesized from 1 μg of freshly prepared total RNA per cDNA reaction mixture in 1× first-strand buffer [50 mM Tris, pH 8.3/50 mM KCl/10 mM $MgCl_2$/1 mM dithiothreitol/1 mM EDTA/0.5 mM spermidine hydrochloride/4 mM sodium pyrophosphate/1 mM dNTPs, containing bovine serum albumin (10 μg/ml) and RNasin (1×10^{-3} unit/μl, Promega)] with oligo(dT) as a primer for RT (275 units/ml, Molecular Genetic Resources, Tampa, FL). A cDNA aliquot was diluted 10-fold and used for PCR. Each PCR mixture contained 5 μl diluted cDNA along with MBP-specific primers (2 μM), actin-specific primers (2 μM), or glyceraldehyde-3-phosphate dehydrogenase (G3PDH)-specific primers (0.7 μM) (Clontech), dNTPs (0.2-mM), 1× PCR buffer [50 mM KCl/10 mM Tris, pH 8.4/3 mM $MgCl_2$ with gelatin (100 μg/ml)], 2.5 units of *Taq* polymerase (AmpliTaq DNA polymerase, Perkin–Elmer/Cetus), and 5-μCi of [α-^{32}P]dCTP (800 Ci/mmol, Amersham; 1 Ci = 37 GBq). The PCR amplification was carried out for 30 cycles of either 30 sec at 95°C, 30 sec at 66°C, 60 sec at 72°C or 30 sec at 95°C, 60 sec at 72°C, depending on the "melting" temperature of the specific primers used. The resulting PCR products were analyzed by electrophoresis in a 4% Hydrolink P600 gel (J. T. Baker), which was dried and exposed to x-ray film. The relative level of each amplified PCR product was quantified with a PhosphorImager (Molecular Dynamics). Although some of the signals may appear to exceed the linear range of x-ray film, they were in the linear range of the PhosphorImager.

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Abbreviations: MBP, myelin basic protein; CNS, central nervous system; PNS, peripheral nervous system; RT, reverse transcriptase; E*n*, embryonic day *n*; P*n*, postnatal day *n*.

*Present address: Department of Molecular Biotechnology, Mailstop FJ-20, University of Washington, School of Medicine, Seattle, WA 98195.

†Present address: The Cedars–Sinai Medical Center, D-3067, 8700 Beverly Boulevard, Los Angeles, CA 90048-0750.

Oligodeoxynucleotide primers were as follows (sequences are shown 5′ to 3′).

MBP 5′ primers	*pro3*	CCGGAGGCCTGGATGTGATG
	start	GCATCACAGAAGAGACCCTCACAGC
MBP 3′ primers	*term*	TCAGCGTCTCGCCATGGGAG
	end7	ATCCAGAGCGGCTGTCTCTCTTCCTCC
Actin 5′ primer	*act122*	GTTGTCGACGACCAGCGCAGCG
Actin 3′ primer	*act123*	CCTCCATCGTGCACCGCAAGT
Embryonic primer	*wz1*	GGAACCGCCCCCACTTGATCCGCCT
G3PDH5′ primer	*G3PDH5′*	TGAAGGTCGGTGTGAACGGATTTGGC
G3PDH3′ primer	*G3PDH3′*	CATGTAGGCCATGAGGTCCACCAC

Western Blots. Brain homogenates were prepared as described (14). Samples were electrophoresed in a 15% polyacrylamide Mini-Protean gel system (Bio-Rad), and the separated proteins were electrotransferred to a poly(vinylidene fluoride) membrane and used immediately with MBP exon-specific antibodies. Exon-specific antibodies were prepared by immunizing rabbits with synthetic peptides that corresponded to various exons of MBP [MBP embryonic exon peptide; RLFSRDAPGREDNTFKDRPSC; MBP exon 1 peptide; ASQKRPSQRSKYLATASTMDC; cysteine (C) was added to the carboxyl termini for conjugation to the carrier protein]. After the blot was blocked with bovine serum albumin, streptavidin, and biotin, protein bands were visualized by reaction with anti-rabbit goat secondary antibody conjugated to biotin and streptavidin conjugated to alkaline phosphatase, followed by a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium. Alternatively, the reactions used anti-rabbit goat secondary antibody conjugated directly to alkaline phosphatase (Boehringer Mannheim and Pierce).

RESULTS

RT–PCR Assay of MBP Isoforms. We used semiquantitative RT–PCR to measure the appearance and subsequent expression of all the MBP mRNA isoforms during mouse development. The sensitivity of RT–PCR was very high, since we were able to detect transcripts in at least 10,000-fold dilution of an aliquot of brain cDNA that was typically used in these experiments (data not shown). Fig. 1*A* represents schematically the expected PCR products when primers are targeted in exons 1 and 7, and their corresponding MBP isoforms. To establish the subsaturation levels of input cDNA and the PCR cycle number, the relative expression level of each MBP isoform was compared with an internal standard generated with actin-specific primers multiplexed with the MBP primers (Fig. 1*B*). In addition, there was no significant change in the amplification efficiency of the actin and MBP primers when the two sets of primers were multiplexed (Fig. 1*C*).

Developmental Expression of MBP Isoforms mRNA in the CNS. Brain RNA from embryonic (E) and postnatal (P) stages of mouse development (days E14, E18, P0, P6, P10, P18, P30, P90, and P162) was converted into cDNA PCR-amplified with MBP-specific and actin-specific primers (Fig. 2). When the relative expression of each MBP mRNA isoform was normalized to actin expression, it was found that the expression pattern of all the MBP mRNA isoforms could be divided into two categories. The first category (21-, 18.5-, 17.2-, and 14-kDa isoforms) began their expression at low levels at various developmental stages and then increased to peak levels at about P18. This peak period lasted until P20 (data not shown). This was followed by decreasing levels on an average of about 50% to a steady-state level where the expression pattern remained constant throughout the life of the animal.

The second category of MBP mRNA isoform expression also began at various developmental stages; however, the expression of these isoforms peaked at about P10 and was undetectable by P18. This category was exemplified by the 17.3-kDa MBP isoform in addition to a minor MBP mRNA isoform, of 19.7 kDa. One of the most interesting patterns of expression was displayed by a member of this second category of MBP mRNA isoform expression that corresponded to a PCR product of about 375 bp. This species was detected as early as E14, much earlier than previously measured for MBP gene expression, and was almost undetectable at P18. Because this MBP mRNA isoform was expressed during embryonic and early neonatal development, it was termed the embryonic–neonatal (E-MBP) MBP isoform.

Given their low signal, we wished to verify that MBP mRNA isoforms were indeed present at these earlier embryonic stages. For this purpose, embryonic and neonatal brain

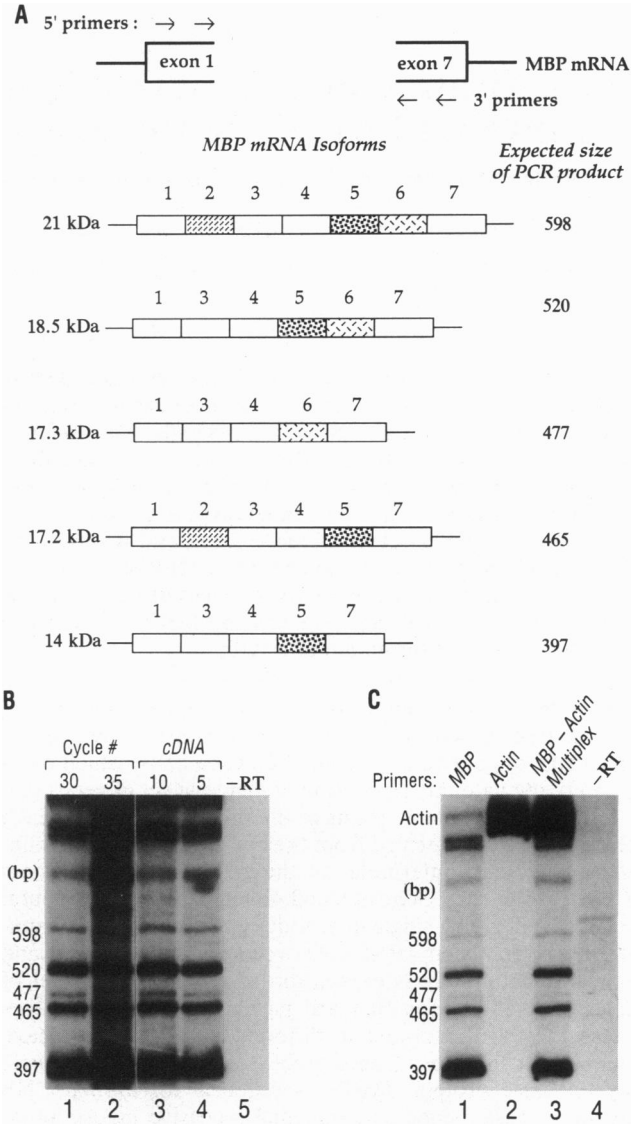

FIG. 1. (*A*) The corresponding molecular mass of each peptide is shown along with the expected size of the DNA product (in base pairs) when exon 1-specific (*pro3*) and exon 7-specific (*term*) primers (arrows) are used in PCR amplification. Hatching and crosshatching indicate differentially spliced exons. (*B*) P18 brain RNA was converted into cDNA and PCR-amplified with [α-^{32}P]dCTP. In lane 2 the cycle number was increased by 5 cycles. In lane 3 twice the amount of cDNA (10 μl) was used as for lane 4 (5 μl). Lane 5 is a PCR amplification with P18 cDNA that was synthesized without RT. (*C*) P18 brain cDNA was PCR-amplified with the primers indicated. Lane 3 is a PCR amplification multiplexed with MBP-specific and actin-specific primers (*act122* and *act123*), producing the five MBP-specific products along with the actin-specific product (1000 bp). Lane 4 is a MBP–actin PCR multiplex using P18 cDNA prepared without RT.

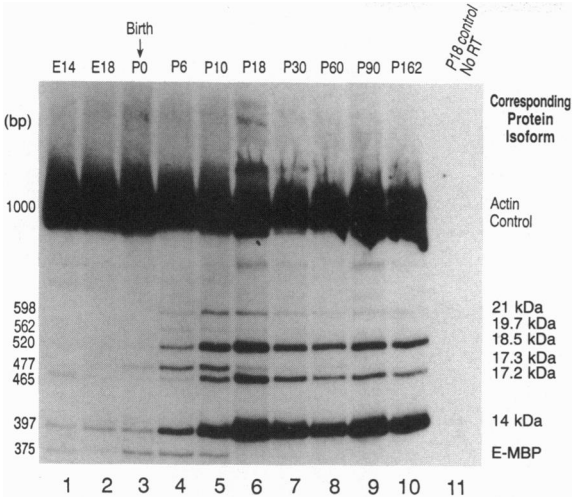

FIG. 2. RNA was isolated from the brains of developmentally staged mice, converted into cDNA and PCR-amplified with [α-^{32}P]dCTP and MBP-specific primers (*pro3* and *term*) and actin-specific primers (*act122* and *act123*). Only the P18 no-RT control is shown.

RNAs were converted into cDNA and amplified without [α-^{32}P]dCTP. An aliquot was then reamplified with [α-^{32}P]dCTP with a second set of nested MBP primers. The resulting PCR products indicated that in addition to E-MBP mRNA, transcripts encoding the 14-, 17.3-, and 21-kDa isoforms were also present in the embryonic brain (Fig. 3, lanes 1 and 2). By the time other MBP mRNA isoforms had reached peak levels of expression, the E-MBP mRNA isoform was almost undetectable (Fig. 3, lane 4). Since the MBP mRNA isoforms began and terminated their expression at different times during development, three distinct patterns of MBP mRNA isoforms were seen in the embryo (21, 17.3, and 14 kDa and E-MBP), neonate (21, 19.7, 18.5, 17.3, 17.2, and 14 kDa and E-MBP), and adult (21, 18.5, 17.2, and 14 kDa). The MBP isoforms expressed in the PNS was also examined. From RNA isolated from neonatal (P6 and P10) sciatic nerves, cDNA was synthesized and PCR-amplified with MBP- and actin-specific primers. All the MBP isoforms

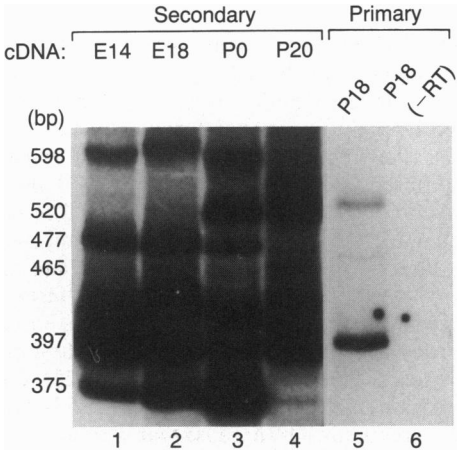

FIG. 3. Brain cDNA was prepared from tissues at various stages of development and PCR-amplified without [α-^{32}P]dCTP using *pro3* and *term* primers. An aliquot was removed from each PCR mixture and amplified with a set of nested primers (*start* and *end7*) with [α-^{32}P]dCTP. A primary amplification of P18 cDNA with [α-^{32}P]dCTP was electrophoresed in an adjacent lane on the gel. MBP isoforms are indicated by molecular size (length, bp) of PCR products.

detected in the CNS were also expressed in the PNS, including the E-MBP isoform (data not shown).

The E-MBP Isoform Contains a Novel Exon. Our laboratory has identified a putative exon located 5′ to the transcription initiation site of the MBP gene, by its similarity between the mouse and shark 5′ regions of the MBP gene. This exon was termed the "wobble zone" (see Fig. 4*A*) (7) and it was postulated that since this region was so highly conserved at the protein level, it was highly likely to represent a coding region. A MBP cDNA clone that also contains sequences 5′ to the transcription initiation site of the MBP gene has also been reported (8). To determine whether the wobble zone was used in any MBP isoforms, RT–PCR assays were performed with wobble zone-specific primers.

When brain cDNA from a neonate was amplified with a wobble exon-specific primer and an exon 7-specific primer, a major PCR product of about 520 bp was produced along with a minor product of about 500 bp (Fig. 4*B*). We have not identified the minor PCR product but suspect that it may be a product of alternative RNA splicing. These two primers were used to produce a nonradioactive amplification product that was isolated as a single 520 bp band from an agarose gel. When the 520-bp PCR product was then reamplified with the exon 1 and exon 7 primers and [α-^{32}P]dCTP, the E-MBP mRNA isoform was detected, indicating that the wobble exon was spliced to only the E-MBP isoform (Fig. 4*B*). E-MBP mRNA isoform expression is restricted to the embryo and the neonate, since the same experiments performed with P30, P60, or P90 brain cDNA yielded no detectable PCR products. Since only the E-MBP mRNA isoform contains the wobble-zone exon, we now refer to this exon as the embryonic exon.

Expression of MBP Peptide Isoforms in the CNS. To ensure that these MBP mRNA isoforms were translated, SDS homogenates of developmentally staged brains were analyzed by Western blots with anti-MBP antibodies prepared against synthetic peptides encoded by specific exons. An exon 1-specific antibody recognized the 21-, 18.5-, 17.2-, and 14-kDa MBP isoforms normally observed in the adult brain (Fig. 5*A*). However, a major peptide of ≈16 kDa was detected from E18 to P6 and was almost completely absent by P10. When the same set of brain homogenates was probed with an antibody prepared against a peptide that corresponded to the translation product of the embryonic exon, a 16-kDa peptide was detected from E18 to P6. This protein began to disappear at P10 and was undetectable by P30 (Fig. 5*B*). The predicted size of a protein translated from the E-MBP PCR product derived from exon 1 and exon 7 primers and added to the translation product of the embryonic exon was estimated to be a protein of about 16 kDa, which matches the size of the peptide observed in the Western blots. Thus, the E-MBP isoform was translated in the developing brain concomitantly to the expression of its putative mRNA. The other MBP isoforms appeared during development coinciding with the beginning of active myelination of the CNS at P6 or later, but not concomitantly with the expression of their respective mRNA isoforms.

Expression of E-MBP in Non-Brain Tissues. When homogenates from a variety of embryonic and neonatal tissues were probed with the embryonic exon-specific antibody, the E-MBP isoform was detected in liver, spleen, testes, and thymus (Fig. 5*C*). Thus, the E-MBP isoform was widely expressed as a 16-kDa peptide in a number of distinct cell types, apart from the nervous system, which are not associated with myelination.

DISCUSSION

Seven MBP mRNA Isoforms Are Differentially Expressed During Development. We have shown that there are two categories of differential expression of MBP mRNA isoforms

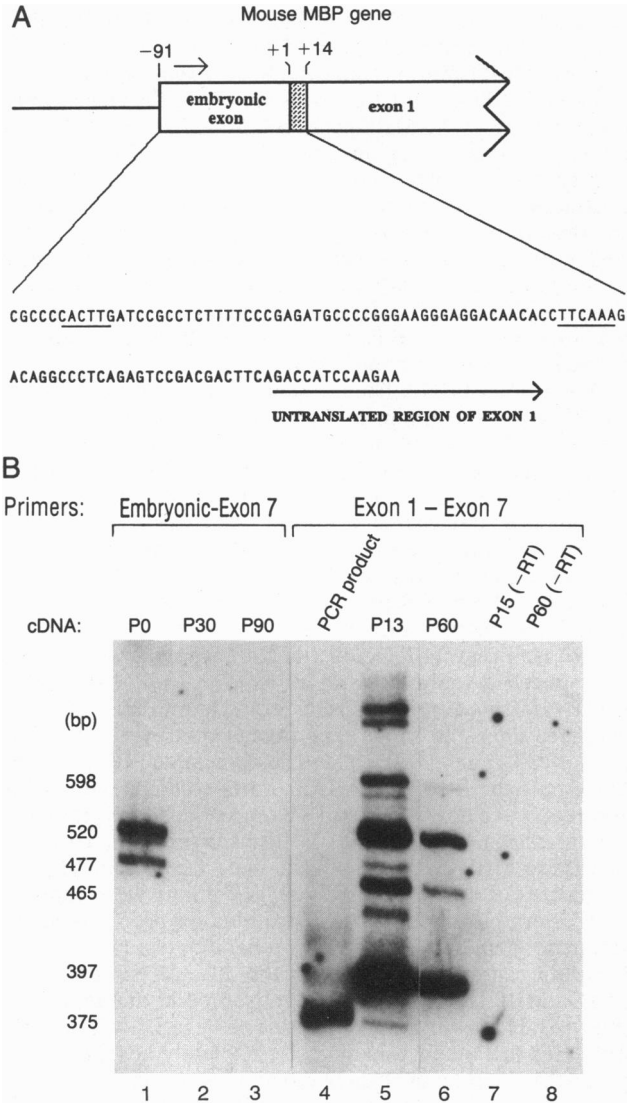

FIG. 4. (*A*) The sequence of the embryonic exon. Underlined sequences are the MBP promoter's putative CAAT and TATA boxes. (*B*) PCR amplification of brain cDNAs prepared from developmentally staged mice. Lanes 1–3 used an embryonic exon-specific 5′ primer (*wz1*) and an exon 7-specific 3′ primer (*term*) with [α-^{32}P]dCTP added. Lane 4 is an amplification of a PCR product that was produced from an amplification with *wz1* and *term*. For lanes 4–7, amplification with the 5′ primer was in exon 1 (*pro3*) and the 3′ primer was in exon 7 (*term*), and [α-^{32}P]dCTP was added. This amplification produced a single band corresponding to the 375-bp E-MBP mRNA isoform (lane 5).

during development. The first category, including mRNAs encoding the 21-, 18.5-, 17.2-, and 14-kDa MBP isoforms, initiates synthesis at varying stages of development, rises to a peak during maximal myelination, at P18, and then declines to approximately half this level for the remainder of the animal's life. The second category of MBP isoforms initiates synthesis prior to birth and is then absent at peak myelination (P18). This differential expression results in distinct combinations of MBP mRNA isoforms expressed in the embryo, neonate, and adult. One of the surprising results of this study was the detection of MBP gene expression in the early embryo. Although other studies have found that the earliest expression of MBP is only in the medulla of the newborn (15, 16), we found expression as early as E14 in all regions of the brain examined (data not shown).

Since MBP mRNAs and the E-MBP protein are expressed in the embryo at a time when oligodendrocytes have not yet

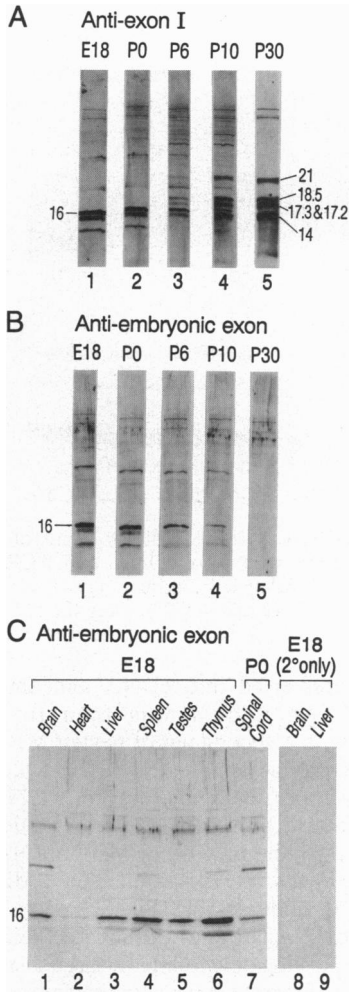

FIG. 5. (*A*) Western blot of brain homogenates from developmentally staged mice probed with an antibody against an exon 1 peptide. Molecular masses (kDa) are indicated at the sides. (*B*) Similar blot probed with an antibody against an embryonic-exon peptide. (*C*) Western blot of various tissue homogenates prepared from either E18 or P0 mice and probed with an antibody against the embryonic exon peptide. As controls, brain and liver lanes that were probed with the secondary antibody only are shown (lanes 8 and 9).

developed, MBP gene expression may be activated in oligodendrocyte precursor cells. CNS progenitor cells would then be committed in the embryo to an oligodendrocyte lineage rather than later during neonatal development. O-2A cells, oligodendrocyte precursors present in the embryo as early as E15, can differentiate *in vitro* into either oligodendrocytes or the nonmyelinating type II astrocytes (17). The embryonic expression of MBP indicates that *in vivo* the embryonic O-2A cells may already be committed to differentiate into oligodendrocytes. Although oligodendrocyte differentiation is thought to proceed cell-autonomously, it has been shown that oligodendrocytes and oligodendrocyte precursor cells are responsive to a number of growth factors (18–21). Thus, MBP gene expression may be activated at low levels in an oligodendrocyte precursor (O-2A cells) that would then await for an induction signal that increases the MBP mRNA levels to those observed in P18 mouse brain.

E-MBP Is Present in Embryonic Tissues and May Have Functions Apart from Myelination. Perhaps the most striking observation was the identification of an isoform expressed only during embryonic and neonatal development. The E-MBP mRNA isoform was detected as early as E14, increased to a maximum level of expression at P6 and then

decreased before active myelination (P18) to undetectable levels. This isoform contains an exon that appears to be expressed only in the embryo and neonate and that is located just upstream of exon 1 in the MBP gene. E-MBP was also expressed at the protein level in a number of embryonic tissues, including the thymus, spleen, and testis. The expression of E-MBP in the embryonic nervous system at a time when there is no myelination or expression of other MBP isoforms and the wide distribution of E-MBP in other embryonic tissues, in addition to the presence of the embryonic exon, are distinct features of the E-MBP isoform. Since E-MBP is present prior to myelination and is expressed in cell types that do not myelinate (e.g., thymus and spleen), it must perform a function other than compaction during myelination. Given its ubiquitous expression in other embryonic tissues, it may be involved in some aspect of differentiation that is not required in the adult. In fact, alternative splicing of exon 2 of the MBP gene may be responsible for the differential intracellular localization of MBP isoforms in the CNS (22).

A number of questions arise about the regulation of MBP expression. MBP transcription must require at least two separate promoters, since the embryonic exon overlaps and extends upstream of the normal transcription start site of the MBP gene (Fig. 4*A*). Transcription of the E-MBP isoform would utilize an upstream initiation site and promoter region to include the embryonic exon in the E-MBP mRNA. Campagnoni *et al.* (23) have shown that additional 5′ exons of the MBP gene produce transcripts that initiate transcription much farther upstream than the previously defined MBP gene and are called Golli-mbp. E-MBP has some of the same exons as the Golli transcript (data not shown). The nature of the production of the six previously identified MBP RNA isoforms poses interesting challenges to the control of alternative forms of mRNA by RNA splicing. Finally, in the embryonic CNS we have detected the 21, 17.2, and 14-kDa and the E-MBP mRNA isoforms; however, during the embryonic phase of MBP expression only the E-MBP mRNA isoform is translated into a peptide. Thus, there is a regulatory mechanism in the embryonic brain that selectively allows only the E-MBP mRNA to be translated. Translation of MBP mRNAs has been shown to be stimulated by steroids through a steroid response element located in the 5′ end of the MBP mRNA, between nucleotides −29 and −73 (24). The E-MBP mRNA isoform contains this sequence and may have additional 5′ sequence(s) that are responsible for its selective translation in the embryo.

The high level expression of the E-MBP isoform peptide, particularly in the thymus and spleen, raises an interesting question in regard to tolerance to MBP (25). It is not known how animals become tolerant to MBP in the embryo, since MBP expression is sequestered by the blood–brain barrier. However, since the E-MBP isoform is widely expressed in the embryo at a time when tolerance mechanisms are most active and in the thymus, which is presumably responsible for tolerance induction, this could be one way of producing tolerance to MBP.

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